

Fusicoccin Activates the Plasma Membrane H⁺-ATPase by a Mechanism Involving the C-Terminal Inhibitory Domain

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Plasma membrane vesicles isolated from spinach leaves incubated with the fungal toxin fusicoccin showed a twofold increase in ATP hydrolytic activity and a threefold increase in H⁺ pumping compared to controls. This increase in H⁺-ATPase activity was largely completed within 4 min of incubation and was not due to *de novo* synthesis of H⁺-ATPase as demonstrated by immunoblotting. Incubation with fusicoccin also resulted in a decrease in the apparent *K_m* for ATP of the H⁺-ATPase from 0.22 to 0.10 mM. The fusicoccin-mediated activation of H⁺-ATPase activity and the accompanying decrease in the *K_m* for ATP are changes very similar to those observed upon trypsin activation of the H⁺-ATPase, where an autoinhibitory domain in the C-terminal region of the H⁺-ATPase is removed. Thus, trypsin treatment of plasma membrane vesicles from control leaves gave a twofold increase in ATP hydrolytic activity and a threefold increase in H⁺ pumping, as well as a decrease in the apparent *K_m* for ATP of the H⁺-ATPase from 0.22 to 0.10 mM. Trypsin treatment of plasma membranes from fusicoccin-incubated leaves did not further enhance the H⁺-ATPase activity, however, and neither was the *K_m* for ATP further decreased. That trypsin really removed a small segment from the fusicoccin-activated H⁺-ATPase was confirmed by immunoblotting, which showed the appearance of a 90-kD band in addition to the native 100-kD H⁺-ATPase band upon trypsin treatment. Taken together, our data suggest that *in vivo* activation of the H⁺-ATPase by fusicoccin proceeds by a mechanism involving a displacement of the C-terminal inhibitory domain.

INTRODUCTION

The plasma membrane H⁺-ATPase plays a major role in the control of cell processes. Using ATP as the energy source, it pumps protons from the cytoplasm to the cell exterior, thus creating an electrochemical gradient across the plasma membrane that constitutes the driving force for nutrient uptake. Extensive acidification of the apoplast is believed to induce cell wall loosening, a prerequisite for cell growth (the "acid growth theory"; for review, see Rayle and Cleland, 1992), and the resulting alkalization of the cytoplasm may be the factor triggering cell division. Because the H⁺-ATPase is involved in nutrient uptake as well as cell growth and division, it is not surprising that its activity is regulated by a number of factors including plant hormones, light, and fungal toxins (for reviews, see Briskin, 1990; Serrano, 1990). However, the mechanism(s) for *in vivo* regulation of the H⁺-ATPase remains to be elucidated.

Incubation with the fungal toxin fusicoccin gives rise to increased acidification of the apoplast in a variety of plant tissues, and this effect has been ascribed to an activation of the plasma membrane H⁺-ATPase (for review, see Marré, 1979). There are no indications for a direct interaction between the toxin and the H⁺-ATPase, and a high-affinity fusicoccin receptor of 30- to 34-kD, distinct from the 100-kD H⁺-ATPase, has been

identified in plasma membrane fractions (De Boer et al., 1989; De Michelis et al., 1989; Meyer et al., 1989). Schulz et al. (1990) have shown that incubation of a pale corydalis cell culture with fusicoccin prior to homogenization leads to a twofold activation of ATP hydrolytic activity measured in the microsomal membrane fraction, and that this ATPase activity has characteristics typical of the plasma membrane H⁺-ATPase. This suggests that the fusicoccin-activated H⁺-ATPase is sufficiently stable in its activated state to withstand membrane isolation, which would render plasma membranes isolated from fusicoccin-incubated tissue a very attractive system for studies on the *in vivo* mechanism for regulation of the H⁺-ATPase.

Recently, it was shown in *in vitro* experiments that the proteolytic (trypsin) removal of a 7- to 10-kD fragment from the C-terminal end of the 100-kD H⁺-ATPase strongly activates both ATP hydrolytic activity and H⁺ pumping by the enzyme (Palmgren et al., 1990a, 1991). It was concluded that a part of the C-terminal region constitutes an autoinhibitory domain of the H⁺-ATPase, and it was suggested that this domain may be the ultimate target for hormones and toxins that function as regulators of H⁺ pumping across the plasma membrane *in vivo* (Palmgren et al., 1991).

Using plasma membranes isolated from spinach leaves incubated with fusicoccin, we now provide evidence that fusicoccin activates H⁺ pumping by a mechanism involving

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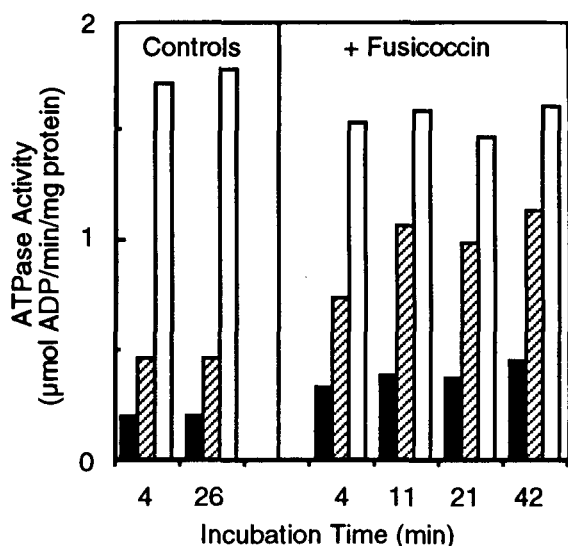


Figure 1. ATPase Activity of Plasma Membranes Isolated from Spinach Leaves Incubated for Different Times with Fusicoccin.

Portions of spinach leaves were cut into pieces, infiltrated under vacuum, and incubated for different times in a medium with 5 μ M fusicoccin (+ Fusicoccin) or without fusicoccin (Controls). Then, plasma membranes were isolated by aqueous two-phase partitioning, and were frozen and thawed twice to produce a mixture of ~40% inside-out vesicles and 60% right-side-out vesicles; the ATP hydrolytic activity of the H^+ -ATPase was determined at pH 7.0. Solid bars denote activity without detergent, i.e., only the activity associated with inside-out vesicles was assayed. Hatched bars indicate activity in the presence of 0.05% of the detergent Brij 58, i.e., total activity. Open bars denote activity in the presence of 0.02% lysophosphatidylcholine, i.e., total activity of the lysolipid-activated enzyme.

the C-terminal inhibitory domain of the H^+ -ATPase, thus demonstrating a physiological role for this domain in the regulation of the H^+ -ATPase.

RESULTS

To study the mechanisms by which fusicoccin activates the plasma membrane H^+ -ATPase, portions of spinach leaves were infiltrated and incubated for different times in a medium with 5 μ M fusicoccin or without fusicoccin (control); the leaves were then homogenized and plasma membranes isolated by aqueous two-phase partitioning. Because plasma membranes obtained by two-phase partitioning are mainly right-side-out vesicles and only inside-out (cytoplasmic side out) vesicles will show activity in the assays for H^+ pumping and ATPase activity (in the absence of detergent), the plasma membranes were frozen in liquid nitrogen and thawed at 20°C twice before use; this treatment gives ~40% inside-out vesicles (Palmgren et al., 1990c).

As seen in Figures 1 and 2, plasma membranes from leaves incubated with fusicoccin showed a twofold increase in ATP hydrolytic activity and a threefold increase in H^+ pumping, respectively, compared to controls. The increase in ATPase activity was observed both in the absence and presence of the detergent Brij 58, thus ruling out the possibility of an artifact arising from differences in the proportions of inside-out vesicles between the preparations. (From the activities \pm Brij 58, the proportion of inside-out vesicles [activity minus detergent/activity plus detergent] can be calculated to between 35 and 45% for the six preparations in Figure 1.)

In the presence of lysophosphatidylcholine, the activities in preparations from control leaves were increased by 280%, whereas the activities of the fusicoccin-incubated material (11- to 42-min incubation) rose by only 40 to 50% (calculated relative to the values obtained with Brij 58, because lysophosphatidylcholine at the concentrations used will also act as a detergent). Thus, plasma membrane preparations from both control and fusicoccin-incubated leaves reached approximately the same level of ATPase activity in the presence of lysophosphatidylcholine, as shown in Figure 1 (open bars).

Lysophosphatidylcholine has earlier been shown to stimulate the H^+ -ATPase activity in isolated plasma membrane vesicles (Palmgren et al., 1988, 1990b; Palmgren and Sommarin, 1989). Furthermore, it has been shown that ATPase activated by lysophosphatidylcholine was not further activated by proteolytic (trypsin) removal of the C-terminal region, and it was suggested that activation by lysophosphatidylcholine involved a displacement of the C-terminal inhibitory domain

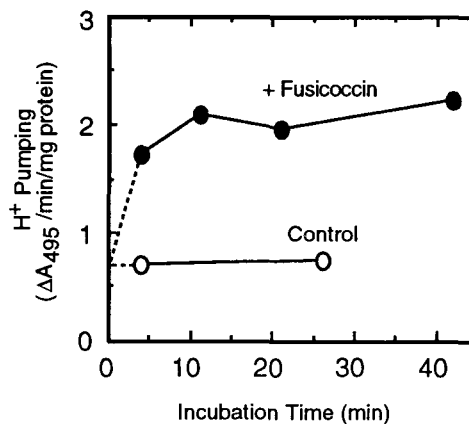


Figure 2. ATP-Dependent H^+ Pumping of Plasma Membrane Vesicles Isolated from Spinach Leaves Incubated for Different Times with Fusicoccin.

Plasma membranes were isolated from spinach leaves incubated for different times in a medium with 5 μ M fusicoccin (closed symbols) or without (open symbols). The plasma membranes were frozen and thawed twice to produce a mixture of ~40% inside-out vesicles and 60% right-side-out vesicles. H^+ pumping by the H^+ -ATPase was determined at pH 7.0 using the Δ pH probe acridine orange.

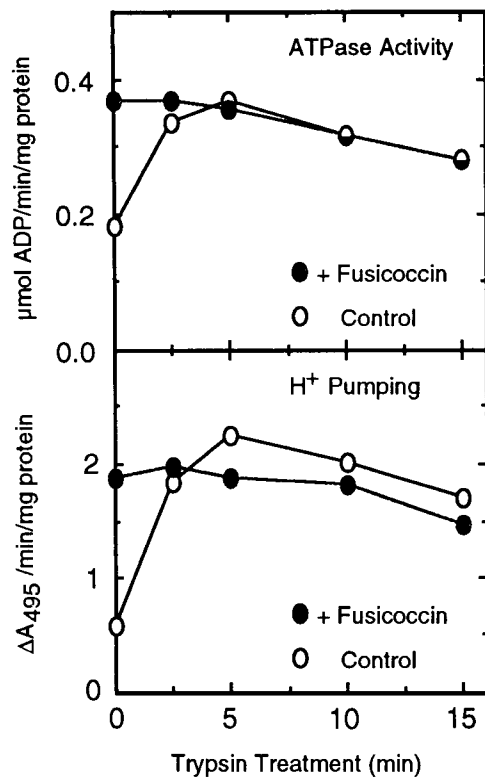


Figure 3. Effect of Trypsin Treatment on ATPase Activity and H^+ Pumping of Plasma Membrane Vesicles Isolated from Fusicoccin-Incubated Leaves and Control Leaves.

Plasma membranes were isolated from leaves incubated for 21 min with $5 \mu M$ fusicoccin (closed symbols) and from control leaves (open symbols). The H^+ -ATPase activity was assayed at pH 7.0 after different times of trypsin treatment of the membrane vesicles.

(Palmgren et al., 1991). As seen in Figure 1, activation by lysophosphatidylcholine was not additive to activation by fusicoccin, which suggests that the latter process may also involve a displacement or removal of the C-terminal inhibitory domain. This prompted us to more closely compare fusicoccin activation of the H^+ -ATPase with in vitro activation by trypsin.

As shown in Figure 3, trypsin treatment of plasma membrane vesicles from fusicoccin-incubated leaves did not increase the ATPase or the H^+ pumping activity of these vesicles; rather, both activities declined slowly with the time for trypsin treatment. This was in contrast to trypsin treatment of plasma membrane vesicles from control leaves, which resulted in a twofold increase in ATPase activity and a threefold increase in H^+ pumping, such that both activities reached the same levels as those of the fusicoccin-activated material. A closer kinetic analysis showed that the apparent K_m for ATP decreased in the control from 0.22 to 0.10 mM upon trypsin

treatment, whereas the K_m for ATP after fusicoccin activation was already 0.10 mM and was not further decreased by trypsin treatment, as shown in Figure 4. That trypsin really removed a small segment of the H^+ -ATPase was confirmed by immunoblotting using an antiserum to the central region of the enzyme. Trypsin treatment resulted in the appearance of a 90-kD band in addition to the native 100-kD H^+ -ATPase band in both the control and the fusicoccin-activated material, as

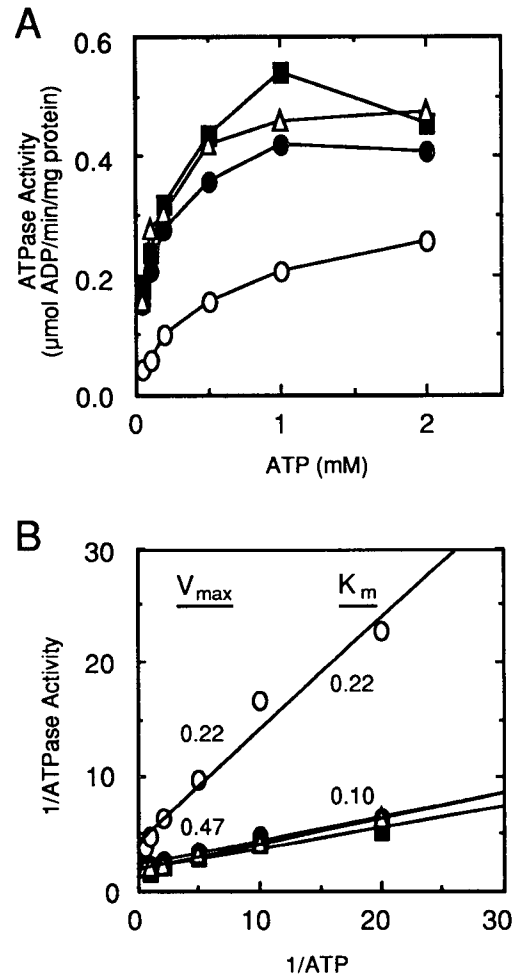


Figure 4. Effect of Fusicoccin and Trypsin Treatment on the V_{max} and on the Apparent K_m for ATP of the ATPase Activity of Spinach Leaf Plasma Membrane Vesicles.

(A) Plot of ATPase activity versus ATP concentration.

(B) Lineweaver-Burk plot of the data in (A).

Plasma membranes were isolated from leaves incubated for 21 min with $5 \mu M$ fusicoccin (Δ , \blacksquare) and from control leaves (\circ , \bullet); the ATPase activity was assayed at pH 7.0 before (\circ , Δ) and after (\bullet , \blacksquare) 5 min of trypsin treatment of the membrane vesicles (compare with Figure 3). V_{max} is given in micromoles of ADP per minute per milligram of protein and K_m is in mM.

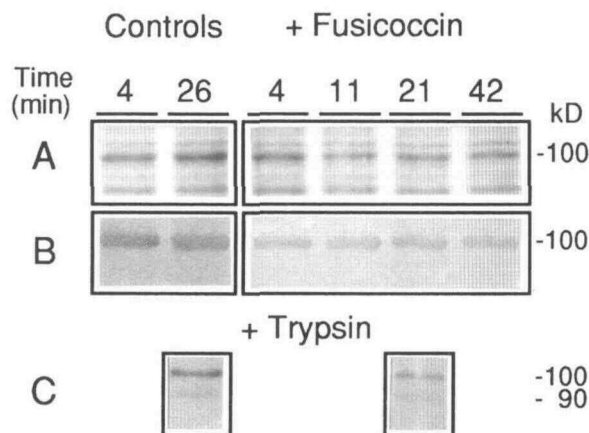


Figure 5. Protein Gel Blot Analysis of the H⁺-ATPase in Plasma Membranes Isolated from Spinach Leaves Incubated for Different Times with Fusicoccin.

Plasma membranes were isolated from spinach leaves incubated for different times in a medium with 5 μ M fusicoccin (+ Fusicoccin) or without (Controls). The plasma membranes were subjected to SDS-polyacrylamide gel electrophoresis (50 μ g of protein per lane), and protein blots were probed with an antiserum to the central region of the H⁺-ATPase.

(A) Protein staining of the part of the gel where the 100-kD H⁺-ATPase is located.

(B) Visualization of the H⁺-ATPase by immunostaining of a protein blot corresponding to the part of the gel shown in (A).

(C) Isolated plasma membrane vesicles were treated with trypsin for 5 min (compare with Figures 3 and 4), and the H⁺-ATPase was visualized by immunostaining of protein blots. Note the appearance of a 90-kD band in addition to the native 100-kD band also seen in (B). Note also that a conversion of more than 40% of the 100-kD band to 90 kD should not be expected, because only the ~40% inside-out vesicles in the preparation expose the C-terminal region of the H⁺-ATPase to the trypsin in the surrounding medium.

shown in Figure 5C. The fact that the fusicoccin-activated enzyme was not further activated by proteolytic removal of a 10-kD fragment and the similar shift to a lower K_m for ATP observed after both fusicoccin and trypsin activation suggest that activation of the H⁺-ATPase by fusicoccin proceeds by a mechanism involving a displacement of the C-terminal inhibitory domain.

As shown in Figures 1 and 2, the fusicoccin-mediated increase in H⁺-ATPase activity was rapid and largely completed within the first 4 min of incubation (shorter incubation times were, for practical reasons, not possible; see Methods). This relatively rapid activation suggests that de novo synthesis of H⁺-ATPase did not contribute to the observed increase in activity. This was supported by the protein staining and immunostaining shown in Figures 5A and 5B; the intensity of the 100-kD H⁺-ATPase band is not increased but rather decreased in samples from fusicoccin-incubated leaves

compared to controls. It is also in agreement with the finding that the plasma membrane preparations from control and fusicoccin-incubated leaves exhibited similar ATPase activities in the presence of lysophosphatidylcholine (Figure 1).

DISCUSSION

Fusicoccin is the major toxin produced by the fungus *Fusicoccum amygdali* and has been found to have a number of physiological effects on virtually all plant species and tissues that have been tested. These effects include stimulation of cell growth, seed germination, stomatal opening, and ion uptake, and they are all linked to an increased acidification of the apoplast, which in turn has been ascribed to an activation of the plasma membrane H⁺-ATPase by the toxin. Thus, fusicoccin either mimics or antagonizes the effects of different plant hormones (for review, see Marré, 1979).

In this report, we show that ATP hydrolysis and H⁺ pumping by the plasma membrane H⁺-ATPase were increased two- and threefold, respectively, when spinach leaves were infiltrated and incubated with a buffer containing fusicoccin prior to homogenization and plasma membrane isolation (Figures 1 and 2). In addition to an increase in the V_{max} of the H⁺-ATPase, in vivo incubation with fusicoccin also caused a decrease in the apparent K_m for ATP from 0.22 to 0.10 mM (Figure 4). These results are similar to those of Schulz et al. (1990); they observed a twofold stimulation of vanadate-sensitive ATP hydrolysis and a reduction to half in the K_m for ATP of that process in a microsomal membrane fraction after incubation of a pale corydalis cell culture with fusicoccin. The result of in vivo activation by fusicoccin resembles that of in vitro activation of the H⁺-ATPase by trypsin, both quantitatively (increase in V_{max}) and qualitatively (decrease in the apparent K_m for ATP), and we show that these activations are not additive (Figures 3 and 4). Similarly, we show that activation by fusicoccin is not additive to in vitro activation by lysophosphatidylcholine (Figure 1). It has recently been demonstrated that activation by trypsin is due to the proteolytic removal of an autoinhibitory domain in the C-terminal region of the H⁺-ATPase and that activation by lysophosphatidylcholine is likely to proceed by a displacement of this inhibitory domain; it has been suggested that this domain may be the ultimate target for hormones and toxins that function as regulators of H⁺ pumping across the plant plasma membrane in vivo (Palmgren et al., 1991). The main conclusion of the present work is that in vivo activation of the plasma membrane H⁺-ATPase by fusicoccin proceeds by a mechanism involving this C-terminal inhibitory domain.

We repeatedly found that fusicoccin stimulated H⁺ pumping more than it stimulated ATP hydrolysis (threefold versus twofold; Figures 1 to 3). This phenomenon was also observed by Rasi-Caldogno and Pugliarello (1985), who found 50 to 130% stimulation of H⁺ pumping but only 20 to 45% stimulation of ATP hydrolysis after incubation of a microsomal membrane

fraction with fusicoccin. This response is very similar to that obtained after trypsin activation of the H^+ -ATPase; activation of H^+ pumping was at least twice that of ATP hydrolysis in both oat root and sugar beet plasma membranes (Palmgren et al., 1990a, 1991). Also in the present study, trypsin treatment stimulated H^+ pumping more than ATP hydrolysis (threefold versus twofold; Figure 3). A stronger stimulation of H^+ pumping compared to ATP hydrolysis was also obtained by micromolar concentrations of lysophosphatidylcholine using oat root plasma membranes (Palmgren and Sommarin, 1989). A possible explanation for this phenomenon is that the acridine orange signal is not linearly related to changes in the ATPase activity. This does not seem to be the case, however, because there is an almost perfect correlation between H^+ pumping measured as acridine orange absorbance change and ATP hydrolytic activity when these activities are altered by varying the ATP concentration (Palmgren et al., 1990a), vanadate concentration, or pH of the assay medium (Palmgren and Sommarin, 1989; Palmgren et al., 1990c). Thus, the data suggest that removal or displacement of the C-terminal inhibitory domain of the H^+ -ATPase indeed causes a change in the coupling between ATP hydrolysis and H^+ pumping such that a more efficient coupling is obtained (Palmgren et al., 1991). If so, this change in coupling may be an important part of the regulatory process.

Recently, it was shown that incubation of abraded maize coleoptiles with auxin (indoleacetic acid) prior to homogenization results in a nearly twofold increase in the amount of H^+ -ATPase polypeptide in the plasma membrane due to an increased rate of synthesis of the enzyme (Hager et al., 1991). This increase in the amount of H^+ -ATPase polypeptide is detectable 10 min after auxin exposure and reaches a plateau after 20 min. However, the fusicoccin-mediated increase in H^+ -ATPase activity was largely completed within the first 4 min of incubation, and no increase in intensity of the 100-kD H^+ -ATPase band could be detected by protein staining or immunostaining (Figures 5A and 5B). This suggests that the increased rates of H^+ -ATPase activity obtained upon fusicoccin activation were not, to any relevant extent, due to de novo synthesis of H^+ -ATPase. This is in agreement with the results of Hager et al. (1991), who found no increase in H^+ -ATPase polypeptide after incubation with fusicoccin. This is also in agreement with the finding that the plasma membrane preparations from control and fusicoccin-incubated leaves exhibited similar ATPase activities in the presence of lysophosphatidylcholine (Figure 1).

One important question that arises is what the precise mechanism is by which fusicoccin activation affects the C-terminal inhibitory domain at the level of the H^+ -ATPase. There are several possible modes of regulation of the plasma membrane H^+ -ATPase involving an autoinhibitory domain, as proposed in the model by Palmgren et al. (1991). Different isoforms may vary in their C-terminal region and exhibit different basal activities. These isoforms can be encoded for by different genes or can be the result of alternative splicing of mRNA from one gene. Activation by fusicoccin, in contrast to activation by auxin

(Hager et al., 1991), is not likely to proceed via regulation at the transcriptional or translational level, because the observed activation by fusicoccin was relatively rapid (Figure 1). Activation via proteolytic removal of the C-terminal inhibitory domain by an endogenous protease can also be excluded, because only a 100-kD H^+ -ATPase band and no 90-kD band was observed after activation with fusicoccin, whereas a 90-kD band appeared upon activation with trypsin (compare Figures 5B and 5C). A possible alternative is that the C-terminal inhibitory domain is displaced after protein kinase-mediated phosphorylation. Thus, the H^+ -ATPase is phosphorylated both in vivo (Schaller and Sussman, 1988) and in vitro, and at least in the latter case by a plasma membrane-bound protein kinase (Bidway and Takemoto, 1987; Schaller and Sussman, 1988). Furthermore, the bacterial toxin syringomycin has been reported to induce phosphorylation of the H^+ -ATPase (Suzuki et al., 1992). The recent findings that a protein kinase inhibitor, K-252a, and fusicoccin induce similar initial changes in ion transport of parsley suspension cells (Kauss et al., 1992) opens the possibility that the H^+ -ATPase is less active when phosphorylated and becomes activated upon dephosphorylation. Another possible mode of activation is that lysophosphatidylcholine, or some other effector, by binding to the H^+ -ATPase induces a conformational change that leads to a displacement of the C-terminal inhibitory domain. This is currently the most attractive hypothesis, because lysophosphatidylcholine has indeed been shown to activate the H^+ -ATPase (Palmgren and Sommarin, 1989; Figure 1), whereas there is no direct evidence that phosphorylation or dephosphorylation affects the activity of the H^+ -ATPase.

In the present study, lysophosphatidylcholine caused a stronger activation of ATP hydrolytic activity than both fusicoccin and trypsin treatments (compare Figures 1 and 3). Thus, it is possible that activation by lysophosphatidylcholine not only involves a displacement of the C-terminal inhibitory domain but also, in some other way, affects the activity of the H^+ -ATPase. However, in vivo activation of a process, such as activation of the H^+ -ATPase by fusicoccin, should not be expected to result in the maximal activation possible. In the same way, trypsin treatment is unlikely to produce maximal activation because trypsin will also degrade and, hence, inhibit the H^+ -ATPase in parallel with activation, as is evident from the latter parts of the activation curves (Figure 3). In contrast, in vitro activation of the H^+ -ATPase by lysophosphatidylcholine may well result in the maximal possible rates of ATP hydrolysis.

Currently, little is known about the components mediating fusicoccin activation of the plasma membrane H^+ -ATPase. A fusicoccin receptor is present in the plasma membrane with an apparent dissociation constant in the low nanomolar range (De Michelis et al., 1989; Meyer et al., 1989) and a molecular mass of 30 to 34 kD (De Boer et al., 1989; Meyer et al., 1989; Schulz et al., 1990). However, the entire signal transduction pathway between the fusicoccin receptor and the H^+ -ATPase remains to be determined. The present approach combining in vivo activation by fusicoccin with in vitro studies on isolated plasma membrane vesicles should be most useful in this work.

METHODS

Plant Material

Spinach (*Spinacia oleracea*) was grown in a greenhouse with supplementary light (23 W/m², 350 to 800 nm; Philips G/86/2 HPLR 400 W). Expanding leaves of 4- to 5-week-old plants were used.

Incubation with Fusicoccin and Isolation of Plasma Membranes

Portions (30 g) of spinach leaves were cut in small pieces (5 × 20 mm) and infiltrated under vacuum with 300 mL of 0.33 M sucrose, 10 mM 2-(*N*-morpholino)ethanesulfonic acid-Tris, pH 6.0, 5 μ M fusicoccin (controls lacked fusicoccin), and then incubated at 20°C for different times. A pH of 6.0 was chosen for the incubation medium because fusicoccin binding is optimal at that pH (e.g., Schulz et al., 1990). After incubation, the leaves were collected on a nylon net, and each portion was homogenized in 50 mL of ice-cooled 0.33 M sucrose, 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)-KOH, pH 7.5, 5 mM EDTA, 5 mM DTT, 5 mM ascorbate, 0.2% bovine serum albumin (protease free, A-3294; Sigma), 0.2% casein (boiled enzymatic hydrolysate, C-0626; Sigma), 0.6% insoluble polyvinylpyrrolidone, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (PMSF was added after filtration of the homogenate). The incubation times indicated above were measured from the start of infiltration to the start of homogenization, and incubation times shorter than 4 min could not be obtained. All procedures following incubation with fusicoccin were at 4°C.

A microsomal fraction (10,000 to 50,000 g pellet) was obtained from the filtered homogenate, and plasma membranes were isolated by aqueous two-phase partitioning using a phase system (8 g final weight) composed of 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 0.33 M sucrose, 5 mM potassium phosphate, pH 7.8, 3 mM KCl, 0.1 mM EDTA, and 1 mM DTT (4°C) (for details, see Larsson et al., 1987). The plasma membranes (~2 mg of protein from each 30-g lot of spinach leaves, the yield was not affected by incubation with fusicoccin) were resuspended in 0.5 mL of 0.25 M sucrose, 25 mM Mops-1,3-bis(tris(hydroxymethyl)methylamino)propane (BTP), pH 7.5, 5 mM EDTA, 1 mM DTT. The plasma membranes were frozen in liquid nitrogen and thawed at 20°C twice before use; this treatment gives ~40% inside-out vesicles (Palmgren et al., 1990c).

Trypsin Activation of the H⁺-ATPase

Trypsin treatment of plasma membrane vesicles was essentially as described by Palmgren et al. (1990a). A small aliquot of plasma membranes (60 μ g protein in 25 μ L) in 0.25 M sucrose, 25 mM Mops-BTP, pH 7.5, 4 mM ATP, 5 mM EDTA, and 2 mM DTT was mixed with an equal volume of 0.25 M sucrose, 25 mM Mops-BTP, pH 7.5, 5 mM EDTA containing 2.5 μ g of trypsin. After incubation at 20°C for the time indicated, the reaction was stopped by the addition of soybean trypsin inhibitor (25 μ g in 25 μ L). In controls (time zero) trypsin inhibitor was added before trypsin.

H⁺ Pumping and ATPase Activity

ATPase activity and H⁺ pumping were monitored simultaneously in the same cuvette (Palmgren and Sommarin, 1989; Palmgren, 1990).

The assay medium contained 10 mM Mops-BTP, pH 7.0, 2 mM ATP, 4 mM MgCl₂, 140 mM KCl, 1 mM EDTA, 1 mM DTT, 20 μ M acridine orange, 0.25 mM NADH, 1 mM phosphoenolpyruvate, 50 μ g/mL pyruvate kinase (in glycerol, 005541; Boehringer Mannheim), 25 μ g/mL lactate dehydrogenase (in glycerol, 003565; Boehringer Mannheim), and 50 to 60 μ g of plasma membrane protein in a total volume of 1 mL. A pH of 7.0 was chosen for the assay medium rather than the more commonly used 6.5, because the physiological pH of the cytoplasm is above rather than below 7 and good fusicoccin activation is observed at pH 7 (Schulz et al., 1990). The plasma membranes were preincubated in the assay medium for 5 min before the reaction was started by the addition of MgCl₂. In this assay, ATP hydrolysis is coupled enzymatically to oxidation of NADH, and the rate of ATP hydrolysis was measured as the absorbance decrease at 340 nm. The H⁺ pumping was measured simultaneously as the absorbance decrease at 495 nm of the Δ pH probe acridine orange. To measure the latent ATPase activity associated with right-side-out vesicles, the detergent Brij 58 was included in the assay at a concentration of 0.05%. Brij 58 was selected in a survey of 42 detergents as a detergent that exposes all latent sites without otherwise stimulating or inhibiting the ATPase activity (Palmgren et al., 1990b). Activation of the ATPase activity by lysophosphatidylcholine was obtained by including 0.02% lysolipid in the assay medium.

SDS-Polyacrylamide Gel Electrophoresis

Each sample of plasma membranes (50 μ g protein, 56 μ L) was supplemented with protease inhibitors (7 μ L of 3 mM tosyl-L-lysine chloromethyl ketone, 0.4 mM leupeptin, 20 mM *p*-aminobenzamidine, and 7 μ L of 10 mM PMSF in ethanol) before mixing with 30 μ L of an SDS-solubilization cocktail. For the trypsin treatments, incubation with protease was carried out in 50 μ L containing 60 μ g of plasma membrane protein as given above, but the reaction was stopped by the addition of 6 μ L of soybean trypsin inhibitor (6 μ g) followed by the protease inhibitor and SDS cocktails, as described above. The samples were incubated with SDS for 15 min at 20°C and then subjected to slab gel electrophoresis essentially according to Laemmli (1970) (total monomer concentration, 8%; crosslinking, 2.7%). Protein was stained with Coomassie Brilliant Blue R 250.

Immunoblotting

The polypeptides were transferred electrophoretically from the polyacrylamide gel to an Immobilon polyvinylidene difluoride transfer membrane (Millipore, Bedford, MA) and reacted with a rabbit antiserum to the plasma membrane H⁺-ATPase using standard procedures. The antiserum was raised against a fusion protein containing the central region of the H⁺-ATPase (Pardo and Serrano, 1989; Palmgren et al., 1991) and was a kind gift from Professor R. Serrano (Universidad Politécnica, Valencia, Spain).

Protein

Protein was measured essentially as described by Bearden (1978), with bovine serum albumin as the standard.

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REFERENCES

- Bearden, J.C., Jr. (1978). Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. *Biochim. Biophys. Acta* **533**, 525–529.
- Bidway, A.P., and Takemoto, J.Y. (1987). Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. *Proc. Natl. Acad. Sci. USA* **84**, 6755–6759.
- Briskin, D.P. (1990). The plasma membrane H⁺-ATPase of higher plant cells: Biochemistry and transport function. *Biochim. Biophys. Acta* **1019**, 95–109.
- De Boer, A.H., Watson, B.A., and Cleland, R.E. (1989). Purification and identification of the fusicoccin binding protein from oat root plasma membrane. *Plant Physiol.* **89**, 250–259.
- De Michelis, M.I., Pugliarello, M.C., and Rasi-Caldogno, F. (1989). Fusicoccin binding to its plasma membrane receptor and activation of the plasma membrane H⁺-ATPase. *Plant Physiol.* **90**, 133–139.
- Hager, A., Debus, G., Edel, H.G., Stransky, H., and Serrano, R. (1991). Auxin induces exocytosis and the rapid synthesis of a high-turnover pool of plasma-membrane H⁺-ATPase. *Planta* **185**, 527–537.
- Kauss, H., Jeblick, W., and Conrath, U. (1992). Protein kinase inhibitor K-252a and fusicoccin induce similar initial changes in ion transport or parsley suspension cells. *Physiol. Plant.* **85**, 483–488.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Larsson, C., Widell, S., and Kjellbom, P. (1987). Preparation of high-purity plasma membranes. *Methods Enzymol.* **148**, 558–568.
- Marre, E. (1979). Fusicoccin: A tool in plant physiology. *Annu. Rev. Plant Physiol.* **30**, 273–288.
- Meyer, C., Feyerabend, M., and Weiler, E.W. (1989). Fusicoccin-binding proteins in *Arabidopsis thaliana* (L.) Heynh. Characterization, solubilization, and photoaffinity labeling. *Plant Physiol.* **89**, 692–699.
- Palmgren, M.G. (1990). An H⁺-ATPase assay: Proton pumping and ATPase activity determined simultaneously in the same sample. *Plant. Physiol.* **94**, 882–886.
- Palmgren, M.G., and Sommarin, M. (1989). Lysophosphatidylcholine stimulates ATP dependent proton accumulation in isolated oat root plasma membrane vesicles. *Plant Physiol.* **90**, 1009–1014.
- Palmgren, M.G., Sommarin, M., Ulvskov, P., and Jørgensen, P.L. (1988). Modulation of plasma membrane H⁺-ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A₂. *Physiol. Plant.* **74**, 11–19.
- Palmgren, M.G., Larsson, C., and Sommarin, M. (1990a). Proteolytic activation of the plant plasma membrane H⁺-ATPase by removal of a terminal segment. *J. Biol. Chem.* **265**, 13423–13426.
- Palmgren, M.G., Sommarin, M., Ulvskov, P., and Larsson, C. (1990b). Effect of detergents on the H⁺-ATPase activity of inside-out and right-side-out plant plasma membrane vesicles. *Biochim. Biophys. Acta* **1021**, 133–140.
- Palmgren, M.G., Askerlund, P., Fredriksson, K., Widell, S., Sommarin, M., and Larsson, C. (1990c). Sealed inside-out and right-side-out plasma membrane vesicles. Optimal conditions for formation and separation. *Plant Physiol.* **92**, 871–880.
- Palmgren, M.G., Sommarin, M., Serrano, R., and Larsson, C. (1991). Identification of an autoinhibitory domain in the C-terminal region of the plant plasma membrane H⁺-ATPase. *J. Biol. Chem.* **266**, 20470–20475.
- Pardo, J.M., and Serrano, R. (1989). Structure of a plasma membrane H⁺-ATPase gene from the plant *Arabidopsis thaliana*. *J. Biol. Chem.* **264**, 8557–8562.
- Rasi-Caldogno, F., and Pugliarello, M.C. (1985). Fusicoccin stimulates the H⁺-ATPase of plasmalemma in isolated membrane vesicles from radish. *Biochem. Biophys. Res. Commun.* **133**, 280–285.
- Rayle, D.L., and Cleland, R.E. (1992). The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* **99**, 1271–1274.
- Schaller, G.E., and Sussman, M.R. (1988). Phosphorylation of the plasma-membrane H⁺-ATPase of oat roots by a calcium-stimulated protein kinase. *Planta* **173**, 509–518.
- Schulz, S., Oelgemoller, E., and Weiler, E.W. (1990). Fusicoccin action in cell-suspension cultures of *Corydalis sempervirens* Pers. *Planta* **183**, 83–91.
- Serrano, R. (1990). Plasma membrane ATPase. In *The Plant Plasma Membrane—Structure, Function and Molecular Biology*, C. Larsson and I.M. Møller, eds (Heidelberg: Springer-Verlag), pp. 127–153.
- Suzuki, Y.S., Wang, Y., and Takemoto, J.Y. (1992). Syringomycin-stimulated phosphorylation of the plasma membrane H⁺-ATPase from red beet storage tissue. *Plant Physiol.* **99**, 1314–1320.